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## PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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INVENTOR(S)					
Given Name (first and middle [if any])		Family Name or Surname		Residence (City and either State or Foreign Country)	
Donald L.		Durden		Indianapolis, Indiana	
<input type="checkbox"/> Additional inventors are being named on the <u>0</u> separately numbered sheets attached hereto.					
TITLE OF THE INVENTION (280 characters max)					
PTEN CONTROLS THE GROWTH AND ANGIOGENIC RESPONSE OF MALIGNANT GLIOMAS					
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input checked="" type="checkbox"/> No.					
<input type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are:					

Respectfully submitted,

  
Signature

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Date May 30, 2000

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Docket No. 099908.1911


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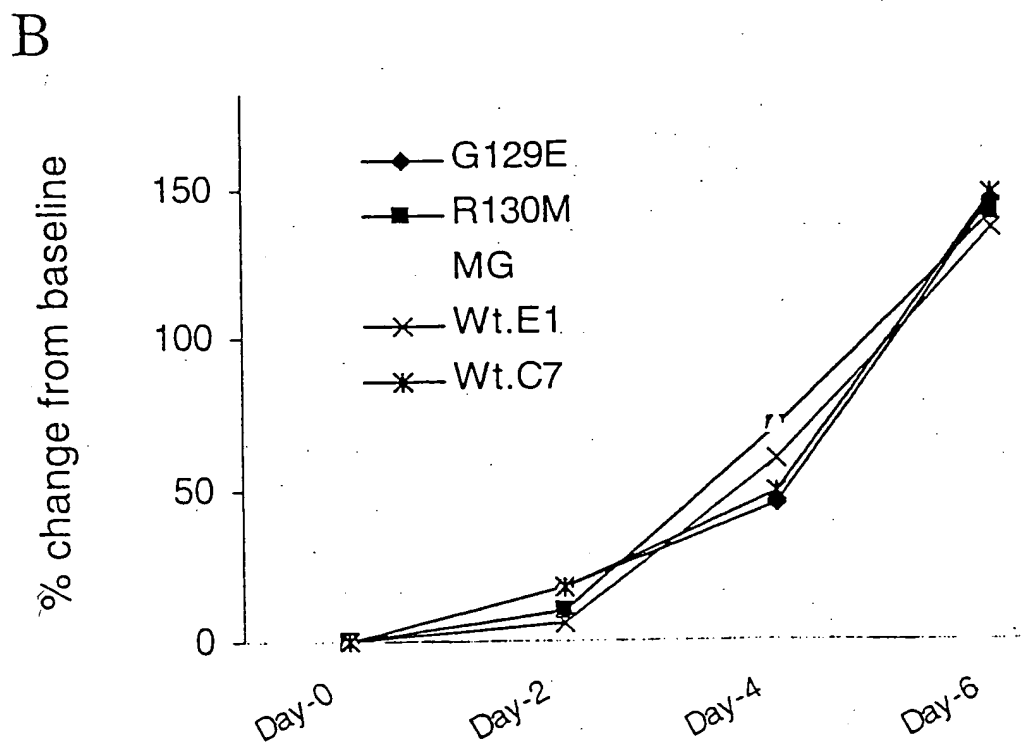
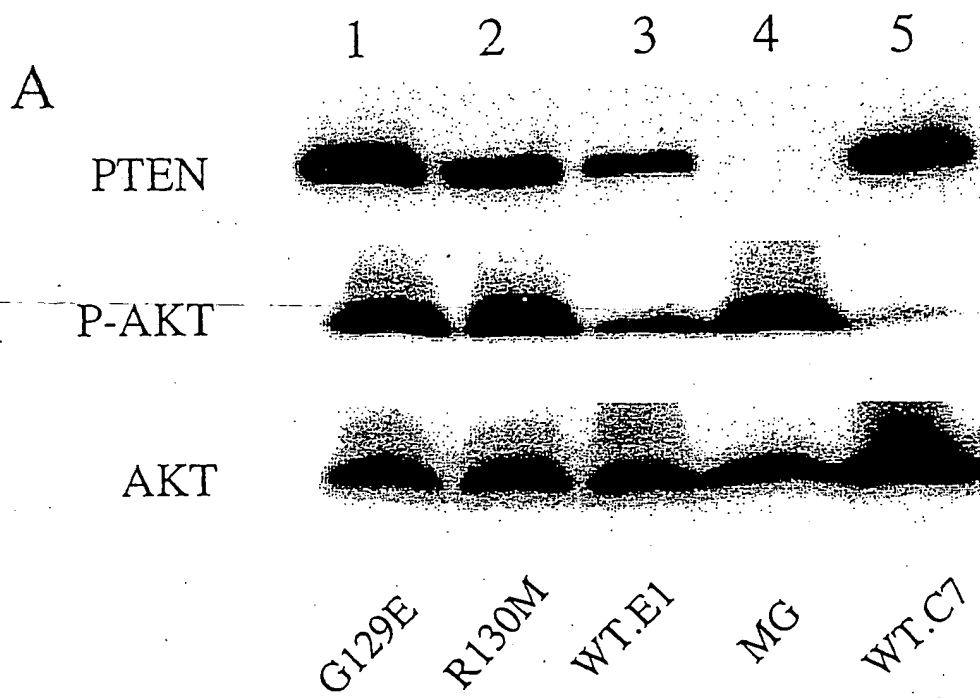
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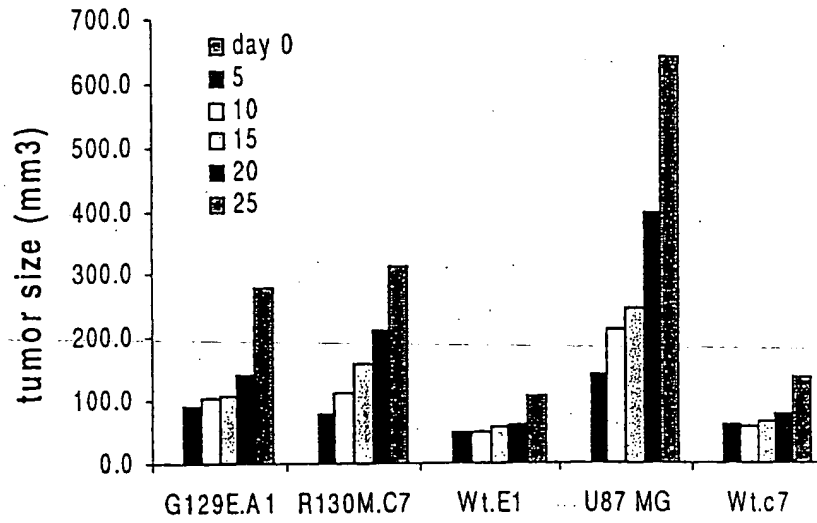
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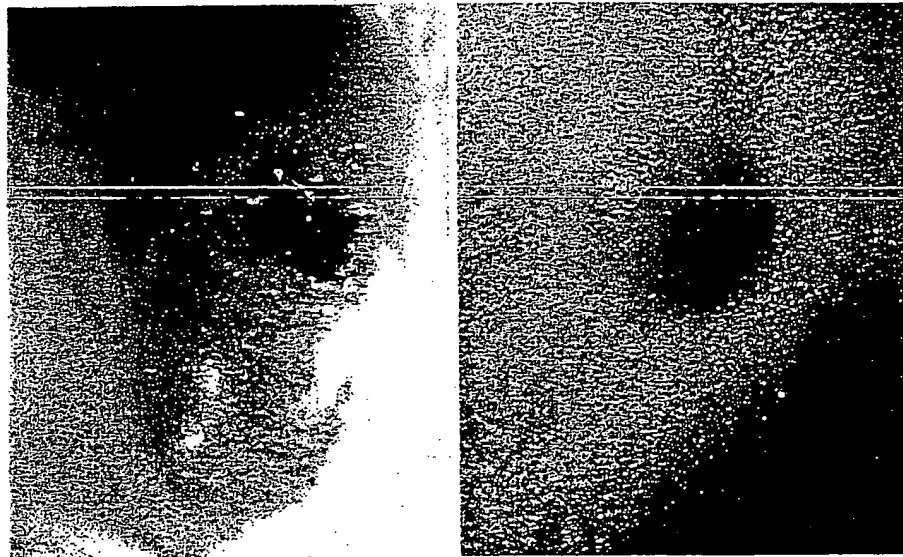
  
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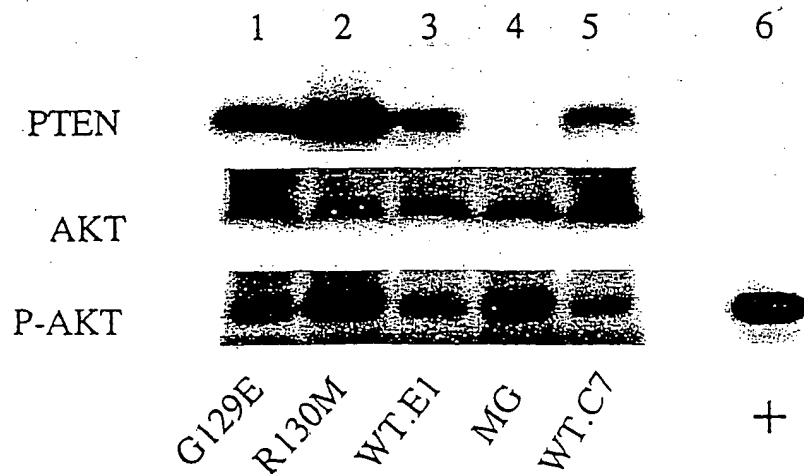
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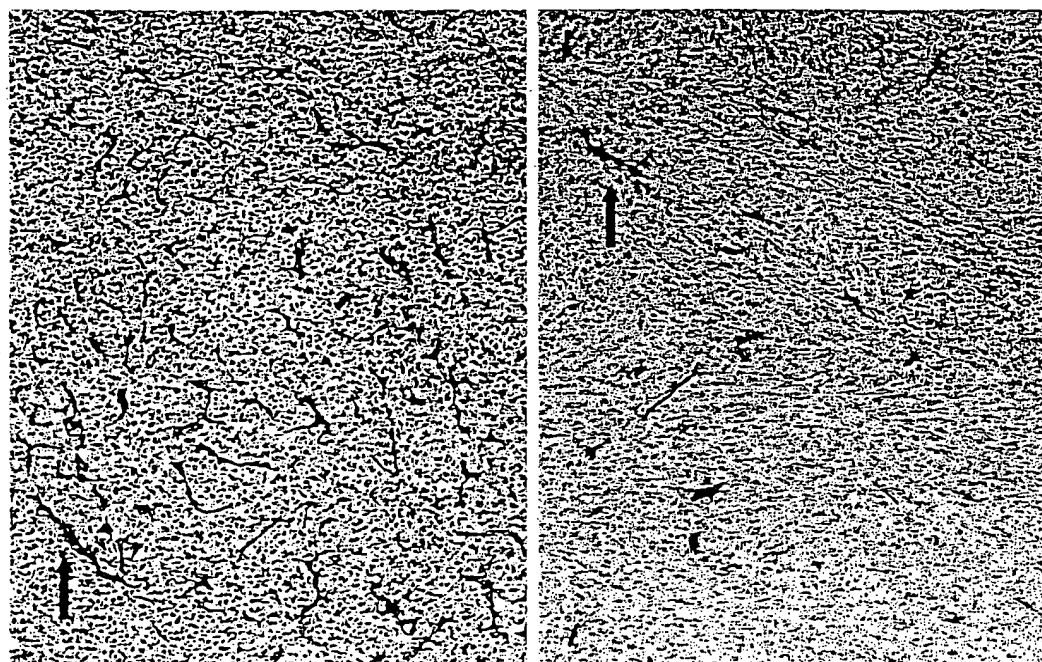


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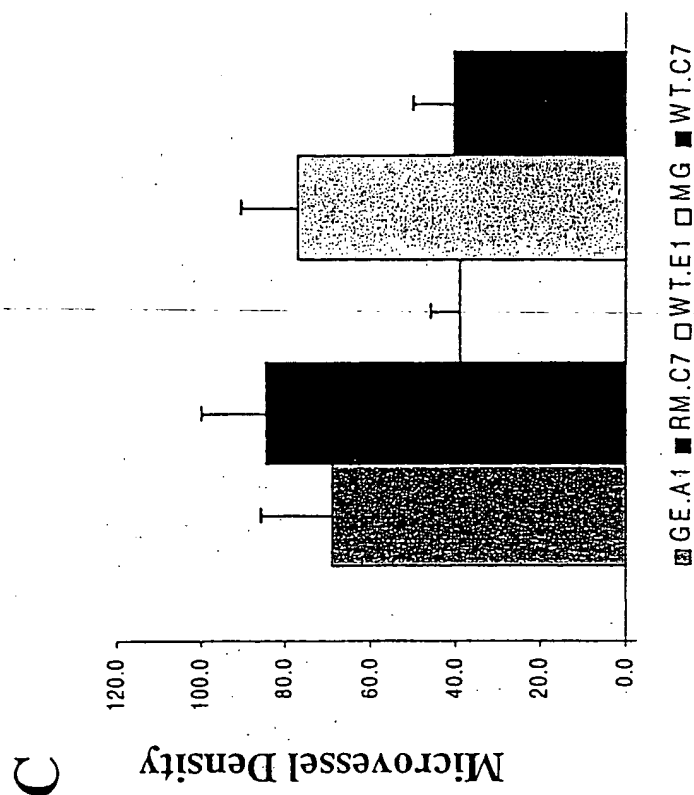
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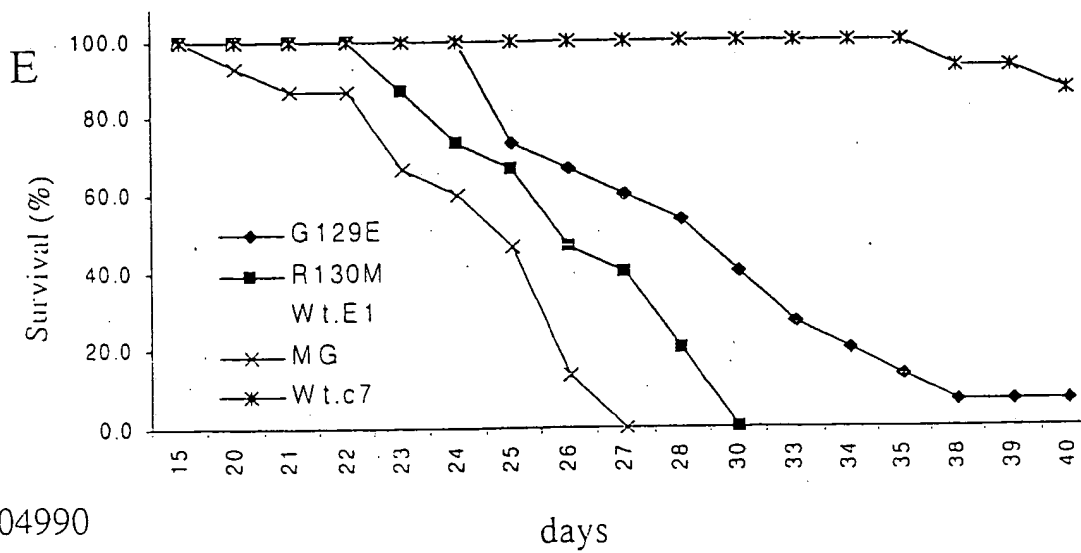
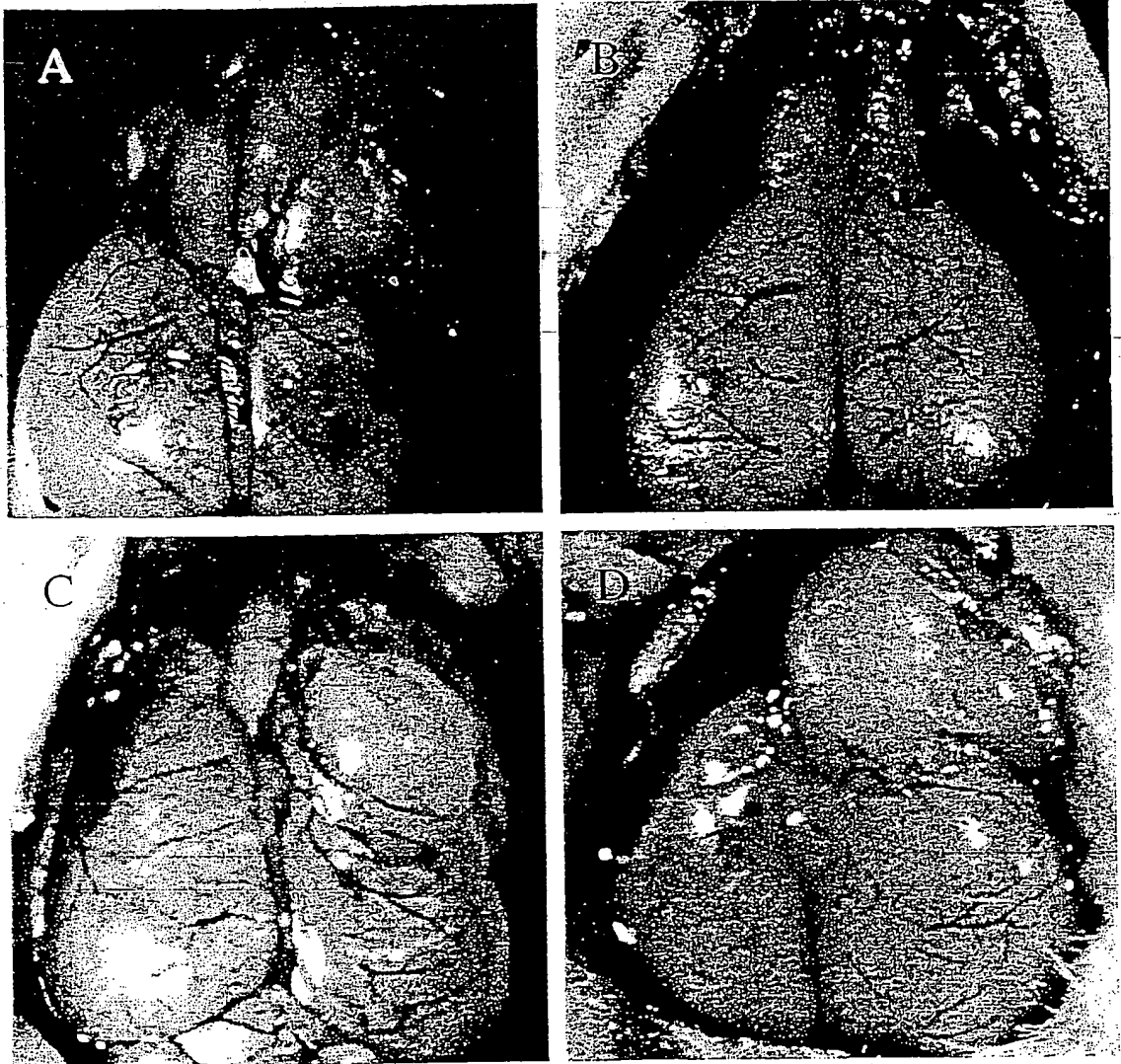




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# PTEN Controls the Growth and Angiogenic Response of Malignant Gliomas

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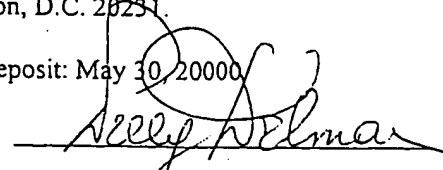
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**Abstract:** Recent evidence suggests that the tumor suppressor gene, PTEN/MMAC/TEP-1 function as a PI-3,4-P<sub>2</sub> and PI-3,4,5-P<sub>3</sub> and a dual specificity protein phosphatase. PTEN is mutated in 42% of human malignant brain tumors and is not mutated in benign gliomas. We hypothesized that PTEN may regulate brain tumor progression *in vivo* and control cell growth by regulating the angiogenic response. U87MG (MG) glioma cells stably transfected with the PTEN cDNA were tested for growth in a nude mouse orthotopic brain tumor model. We observed that the introduction of wild type PTEN resulted in decreased growth of these tumors *in vivo* and prolonged survival in mice implanted intracranially with this tumor. The dramatic increase in tumor growth *in vivo* correlates with augmented levels of phosphorylated AKT kinase within the PTEN deficient tumor mass. PTEN minus and PTEN reconstituted tumor tissue displayed marked differences in angiogenic activity as determined by microvessel density. Augmented levels of angiogenesis was correlated with levels of phosphorylated AKT within the tumor tissue and represent the first direct evidence that PTEN/AKT controls tumor-induced angiogenesis and the progression of gliomas to a malignant phenotype.

The phosphorylation of proteins and lipids is regulated in mammalian cells by kinases and phosphatases (1). The tumor suppressor gene PTEN/MMAC/TEP-1 (hereafter termed PTEN) has been shown to function as a dual specificity phosphatase to control phosphoinositide and protein phosphorylation events within the cell. It is mutated in 40-50% of high grade gliomas as well as prostate,

endometrial, breast, lung and other tumor types (1). Earlier work demonstrated that chromosome 10 was partially or entirely deleted in 90% of glioblastoma multiforme derived cell lines, the most aggressive type of brain tumor diagnosed in humans (2). Subsequently the PTEN gene was identified as a gene located on short arm of chromosome 10 (10q23) which is mutated in these cell lines and in primary tumors and other malignancies (3, 4, 5). PTEN is a 55 kilodalton protein containing an N terminal tensin homology and a C terminal catalytic and lipid binding C2 domains and an end terminal PDZ motif. It contains a consensus YXXVKR catalytic signature motif which is found in all protein tyrosine phosphatases (6). This enzyme, unlike other dual specificity phosphatases, preferentially dephosphorylates phosphoinositides at the D3 position of the inositol ring (7, 8). Current data suggests that PTEN principally regulates the PI-3 kinase/AKT kinase pathways as well as controls protein tyrosine kinases and other serine/threonine kinases through protein dephosphorylation (1, 9). Several observations established a link between the PI-3K/AKT pathway and PTEN in tumor cells which carry PTEN mutations (10, 11). Despite progress in the understanding of PTEN biochemistry, the role of PTEN in tumor progression as it relates to cell growth, angiogenesis and/or survival remains unclear.

The discovery that PTEN was mutated in several rare autosomal dominant cancer syndromes, Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome provided further evidence that PTEN is a tumor suppressor gene (12). A missense mutation (G129E) was confirmed in 2



kindreds of Cowden disease where a glycine was substituted with a glutamic acid residue. This mutation was shown not to affect protein phosphatase activity but to obliterate PI-3 phosphatase activity suggesting a role for this enzymatic activity in tumor suppression (10). More recent data demonstrate that PTEN mutations occur in pediatric high-grade gliomas and correlate with poor survival (13). Data from the PTEN knockout mice demonstrated a requirement for PTEN in normal development and the heterozygous disruption of PTEN confirmed its role in the control of tumorigenesis (14, 15, 16).

PTEN is mutated in late stage tumors and as brain tumors progress from grade I/II tumors to malignant grade III and IV tumors *in vivo* (13, 17). Tumor progression is associated with acquisition of the angiogenic phenotype with increase in microvessel density and increased invasion of tumor cells into brain parenchyma (18, 19). Hsu et al demonstrated in glioma cell lines that the transfer of wild type chromosome 10 could reverse the angiogenic phenotype of malignant gliomas and prevent growth of tumors in nude mice (20). These combined observations led us to hypothesize that PTEN may control the angiogenic response and contribute to the high mortality associated with malignant brain tumors.

Angiogenesis, the formation of new blood vessels from existing vascular structures, is important for wound healing and tumor growth beyond 2 mm<sup>3</sup> (21). For tumor growth to occur, tumor dormancy must be broken, an event termed the

"angiogenic switch" (22). During angiogenesis endothelial cells are induced to degrade the basement membrane of existing vessels, break away and migrate to the site of the tumor, where they proliferate to form linear structures from which they differentiate to form lumens. Factors that control angiogenesis include growth factors, matrix metalloproteinases, plasminogen activators and integrins  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$  and  $\alpha_5\beta_1$  (23). Malignant tumors tend to induce a more robust angiogenic response as compared to their benign counterparts. Malignant brain tumors are classified histopathologically by presence or absence of high microvessel counts (microvessel density). Several lines of evidence suggest a role for VEGF and the VEGF receptor 1 (Flt-1) in human brain tumor angiogenesis (24) and others have reported that hypoxia induced VEGF is modulated via PI-3 kinase/AKT pathway involving the HIF1 transcription factor (25, 26). Jiang et al performed experiments in the chicken chorioallantoic membrane (CAM) model demonstrating that PI-3 kinase pathways may regulate angiogenesis and VEGF expression in endothelial cells. Jiang et al demonstrated that the overexpression of PTEN using an avian retroviral vector inhibited angiogenesis within the yolk sac of the chicken embryo (27). In these experiments, the expression of PTEN would have been throughout all CAM tissues making it difficult to ascribe the effect of PTEN expression to one specific cell type. Giri et al recently observed that prostate tumor specimens from patients with PTEN mutations have higher microvessel counts as compared to tumors with wild type PTEN expression (28). Whether PTEN is causally linked to

tumor cell induction of angiogenesis remains unproven. The U87MG cell line is derived from a patient diagnosed with glioblastoma multiforme, a highly malignant and uniformly fatal brain tumor. This tumor and other human glioblastomas and glioblastoma cell lines have since been shown to contain a genetic mutation in both PTEN alleles (MG cells have homozygous mutation in exon 2 resulting in null genotype). In light of these observations, we reconstituted the PTEN gene in the parental U87MG (MG) cells to determine the role of PTEN in tumor growth, angiogenesis and brain-tumor related survival.

To determine if PTEN exerts control over the angiogenic and/or growth process of glial tumors, we developed an orthotopic brain tumor model where the parental MG cells were genetically manipulated *in vitro* and then stereotactically injected into the frontal cerebral cortex of nude mice. In this model 100% of mice implanted with the parental MG tumor display a highly invasive and angiogenic pattern of brain tumor growth. Within 25 days, the mortality of mice implanted intracranially with this tumor is 100% (data not shown). The parental MG cells were stably transduced with retrovirus encoding the wild type PTEN gene or specific mutants of PTEN as described (10). Stable clones of MG cells expressing the wild type PTEN (WT) or PTEN mutants (mutation at G129E, which is dead for PI-3 phosphatase activity or mutation R130M, which is dead for all catalytic activity) were established by G418 selection. Tumor cells were characterized biochemically for levels of activated AKT, *in vitro* growth and PTEN expression pattern (Figure 1). Anti-PTEN blots confirmed that the parental MG

cells do not express PTEN and that PTEN transduced MG cells express significant and comparable amounts of PTEN protein. We also demonstrated *in vitro* that wild type PTEN expression at levels similar to a PTEN positive mouse brain lysate suppressed the activated state of AKT, which is observed in MG, cells deleted in PTEN (Figure 1A). Expression of PTEN mutants deficient in total phosphatase (R130M) or PI-3 phosphatase (G129E) were observed to have similar phospho AKT levels suggesting that the control of PIP3 in these cells is through PI-3 phosphatase activity. The *in vitro* growth of the different PTEN expressing MG cell lines was similar in 2, 5 and 10% fetal bovine serum (data not shown) (Figure 1B) a result which supported the further comparison of these cell lines in our *in vivo* models.

Next, we determined the *in vivo* behavior of the PTEN minus and PTEN reconstituted glioma cells. We implanted athymic nude mice both subcutaneously and by intracranial injection. The subcutaneous tumors allowed us to carefully monitor size of tumor and to perform biochemical analysis of tumor tissue for PTEN expression and levels of AKT activation without significant contamination from other mouse tissues (Figure 2 and 3). The results show a dramatic difference in growth rate of MG tumors as compared to wild type PTEN reconstituted tumors (Figure 2A-B). Average tumor volume of MG tumor on day 25 after implantation was  $848 \pm 203$  as compared to  $91 \pm 27$  for PTEN reconstituted tumors ( $p < .0001$ ). Tumor tissue blocks were processed for H and E stains to confirm that greater than 95% of tissue was tumor cells free of dermal

or subdermal tissue (data not shown). Importantly, we compared anti-PTEN Western analysis of tumor tissue to numerous normal tissues within the athymic nude mouse. Using our anti-PTEN antisera, we were able to detect the expression of PTEN in all tissues with exception of skeletal and heart muscle and no PTEN was detected in the parental MG tumor tissue (data not shown). These results indicate that the tumor tissue sampled predominantly represents tumor cell derived proteins. The pattern of PTEN expression in the subcutaneous tumor was compared to that observed in cell lines grown *in vitro* (Figure 1A to 2C). Next we compared the phosphorylation state of AKT in the tumor tissue (Figure 2C). This allowed us to biochemically evaluate, correlate and potentially identify the effectors controlled by PTEN within the tumor mass. The data are consistent with recent *in vitro* observation of Zundel et al (29), suggesting that PTEN controls the phosphorylation state of AKT through the action of PI-3 dephosphorylation. Our data confirms this biochemically *in vivo* within the tumor.

To assess the angiogenic potential of the parental MG cells versus MG cells reconstituted with PTEN or PTEN mutants, we stained cryostat sections from subcutaneous tumors for CD31 (PECAM). This is the marker for the angiogenic endothelium used to measure the microvessel density (MVD) of these tumors. Microvessel density was assessed from multiple digitized images of CD31 stained tumor tissue at 100x magnification and counted blindly for number of CD31 positive microvessels per unit surface area as described (19) (Figure 3). The results demonstrate that PTEN reconstitution suppresses the angiogenic

response *in vivo*. A comparison of MVD in wild type PTEN transduced tumors ( $38 \pm 7$ ) to MG tumors ( $77 \pm 13$ ) reveals difference ( $n=5$ ,  $p < .001$ ). The microvessel density of tumors derived from MG cells transduced with catalytically dead PTEN (R130M or G129E) ( $84 \pm 15$  and  $69 \pm 16$  respectively) were not significantly different ( $p > .05$ ) from the parental MG cell line (Figure 3A-C). The phospho-AKT levels within the tumor mass *in vivo* suggest a mechanistic link between the loss of PTEN PI-3 phosphatase function, the phosphorylation of AKT and the angiogenic phenotype within the tumor. It is noted that angiogenesis is not completely abrogated by PTEN replacement (Figure 3C) suggesting a role for other factors in tumor induced angiogenesis. Our data provide the first direct evidence that PTEN controls the angiogenic behavior of tumor tissue *in vivo*.

To determine if PTEN status plays a role in brain tumor-related survival, MG cells transduced with wild type PTEN or specific mutants of PTEN were implanted under stereotactic control into the right frontal lobe of nude mice (Figure 4A-E, see arrow for site of implantation). The results indicate that the reconstitution of wild type PTEN in MG cells suppresses the capacity of these tumors to grow in the brain. PTEN reconstitution was observed to suppress the malignant potential of the MG cells in our orthotopic animal model as assessed by 90% survival at 40 days as compared to 100% mortality of mice implanted with MG at 27 days (Figure 4E). PTEN reconstituted tumor cells were noted to grow more slowly when implanted in frontal lobe (Figure 4) and remain circumscribed to that area of brain (data not shown). Parental MG cells transfected with mutants of PTEN,

which lack inositide phosphatase activity (G129E) or catalytically inactive PTEN (R130M) displayed a phenotype similar to the PTEN negative MG cells. Tumors derived from MG cells transduced with PTEN which lacks PI-3 phosphatase activity has a slightly prolonged survival (50% at day 30), however all animals died by day 40. These data suggest that loss of PI-3 phosphatase catalytic activity alone is sufficient to convert MG tumor cell to an angiogenic (Figure 3) and malignant glioma (Figure 4A-E).

Herein, we report the first direct evidence that the PTEN tumor suppressor gene regulates brain tumor growth and angiogenesis *in vivo*. Moreover the PTEN mutation in these malignant tumors is an important determinant of their malignant behavior *in vivo*. This model will be useful to determine other downstream physiologic targets, which are negatively regulated by PTEN (extracellular matrix-integrin interactions, angiogenic target genes, and response to PI-3 kinase inhibitors) as part of progression to the malignant angiogenic brain tumor phenotype. Lastly, these data suggest a potential therapeutic benefit for PI-3 kinase inhibitors in treatment of malignant gliomas.

### Figure Legends

**Figure 1 Stable expression of PTEN and PTEN mutants in U87MG (MG) cells regulates AKT. (A)** Cell lysates from the U87MG (MG) cell line and MG cells infected with a retroviral vector for PTEN expression were resolved by SDS-PAGE, equal amounts of proteins were loaded per lane and immunoblotted

with antisera to PTEN, phospho-AKT and total AKT, and visualized by enhanced chemiluminescence. The basal levels of PTEN (top), phosphorylated AKT (ser 473) (middle) and total AKT (bottom) are shown. The status of the PTEN gene in each cell line was designated as: WT.E1 and WT.C7 express wild type PTEN, R130M and G129E are mutated PTEN, R130M is inert as both a protein and a lipid phosphatase. G129E is the allele, that can dephosphorylate acidic phosphopeptides, but cannot dephosphorylate lipid substrate, PIP3. The U87MG (MG) cell line is the parental cell line isolated from a human glioblastoma multiforme patient. (B) Comparison of *in vitro* growth of MG cells transduced with mutants of PTEN. Equal number of cells ( $1 \times 10^5$ ) were incubated in RPMI + 10% FBS for different times and cell numbers were quantitated by direct cell counting.

**Figure 2. Effects of PTEN on growth of U87MG cells *in vivo*.** (A) Pattern of *in vivo* cell growth. In order to determine the cell growth rate *in vivo*, equal amount of cells ( $5 \times 10^6$ ) from each cell line were implanted at the right ventral flank by subcutaneous injection. The formation and growth of the subcutaneous tumor was monitored and the size of the tumor was determined by the three dimensions measurement at the times indicated. The volume of the subcutaneous tumor was determined by measuring the three dimensional diameter. Data were analyzed by Student t-test and differences were significant comparing the PTEN deficient (MG, R130M, G129E) to the wild type PTEN (WT.E1, WT.C7),  $n=5$ ,  $p<.0001$  B) Stereophotography of subcutaneous tumor



sites in mice implanted with the parental MG tumor, PTEN minus (left panel) versus wild type PTEN reconstituted tumor cells (right panel). These tumors represent 25 and 42 days after implantation for PTEN minus versus PTEN reconstituted tumors, respectively. C) Western blot of cryostat tissue sections from subcutaneous tumor for the expression pattern of PTEN, AKT and phosphorylated AKT. Frozen tissue sections were solubilized in Laemmli sample buffer, total protein was quantitated and equal protein was loaded on SDS PAGE. The data shown are representative of tissue analysis from 5 animals per experimental group.

**Figure 3. PTEN suppresses angiogenesis.** Immunohistochemical analysis with CD31 antibody staining to view the angiogenesis response within the parental MG tumor and PTEN reconstituted tumors, implanted into the subcutaneous tissue. We show CD31 staining on PTEN minus MG tumor, (A) versus wild type PTEN reconstituted tumor tissue, (B). In PTEN minus and tumors expressing mutants of PTEN, there are more new vessels formed (angiogenesis) (upper panel, arrow indicated) than in wild-type PTEN reconstituted tumor (lower panel), indicating the PTEN has direct influence on angiogenesis during tumor growth. C) Microvessel density (MVD) counts were performed on tumor tissue stained with anti-CD31 antibody as described to determine effect of expression of PTEN and specific PTEN mutants on tumor induced angiogenesis. Bars represent SD. Statistical analysis by Students t-test demonstrate significant difference between MVD of PTEN null and PTEN

catalytic mutants as compared to wild type PTEN reconstituted tumors,  $n=5$ ,  $p<.001$ .

**Figure 4. Effects of PTEN reconstitution on survival in an orthotopic brain tumor model.** Equivalent number of parental MG or MG reconstituted with wild type or mutant alleles of PTEN (see legend) ( $1 \times 10^6$  cells) were implanted in right frontal lobe of nude mice. Cells were cultured in fresh medium for 24 hours and harvested, adjusting the cell concentration to  $1 \times 10^6$  in 10  $\mu$ l of RPMI medium. Mice under general anesthesia were placed into the stereotactic devise (model 963, Kopf, Tugunga, CA). Stereotactically controlled drill assembly was used to provide a 0.3 mm deep and 0.8 mm/diameter hole in cranium at a position 0.5 mm anterior and 1.2 mm lateral to the bregmal anatomical landmark. Tumor cells ( $1 \times 10^6$ ) were introduced slowly through a 10  $\mu$ l Hamilton syringe at a depth of 2.5 mm at a rate of 2  $\mu$ l per minute. We then slowly removed the needle at a rate of 0.5 mm/min. After needle removal we seal the hole with bone wax and close incision with wound clip. Stereophotography of whole brains (A-D) from mice implanted with wild type PTEN reconstituted tumor (day 42) (B); tumor implantation site is shown by position of arrow in the wild type PTEN reconstituted tumor, (B) (magnification x 20). Whole brain from PTEN null MG tumor (day 25) (A) or catalytically dead mutant of PTEN (R444M)(day 30) (C) or mutant of PTEN without PI-3 phosphatase activity (G129E)(day 32), (D). (E) Kaplan-Meier plots for survival in mice implanted with PTEN minus or parental MG cells transduced with mutants of PTEN as shown. Survival data represents 5

animals per experimental group.  $n=5$ ,  $p < .0001$  for difference observed between the PTEN + and PTEN – groups for survival.

#### References and Notes.

We would like to dedicate this work to the memory of our patients Ross Feikls and Kandra Gresik who died of malignant brain tumors; for their bravery and inspiration given to us by them. We would like to acknowledge the excellent technical assistance of Lee Ann Balderidge, Angela Hatten and Kayvon Izadi. We would like to thank Drs. Carrie Phillips and Biagio Azarelli for there support. Funding for this work was from grant from NIH RO1-CA75637 and ACS RPG-98-244-01-LBC to DLD and grants number 6666 and 4444 to NKT and MHW, respectively.

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